"STUDIES ON THE MICROBIAL DEGRADATION OF CARBOHYDRATES IN ASIAN HONEY BEE GUT"

PhD FINAL SYNOPSIS

Department of Studies & Research in Biochemistry

Mangalore University

In Fulfilment of the requirement for the degree of

Doctor of Philosophy in Biochemistry

Submitted by

Mr. GANESHPRASAD D. N.

(Reg.No: MU/EXB/Ph.D./CR.115/Bio-chem./2020-21/E.13)

Under the Guidance of

Dr. SNEHA RANI A. H.

Assistant Professor, Research Supervisor

DEPARTMENT OF STUDIES & RESEARCH IN BIOCHEMISTRY
JNANA KAVERI PG CENTRE, CHIKKA ALUVARA, KODAGU -571232

August 2022

Name of the Candidate: Mr. Ganeshprasad D.N.

Name of the Guide: Dr. Sneha Rani A.H.

Designation: Assistant Professor
Department of Studies in Biochemistry
Jnana Kaveri P.G. Centre, Chikka Aluvara
Mangalore University

Registration No: MU/EXB/Ph.D./CR.115/Bio-chem./2020-21/E.13

Date of Registration: 18-02-2017

Title of the Thesis: "STUDIES ON THE MICROBIAL DEGRADATION OF CARBOHYDRATES IN ASIAN HONEY BEE GUT"

Studies on the Microbial Degradation of Carbohydrates in Asian Honey Bee Gut

Introduction

Gut microbiota research is an emerging discipline that aims to learn more about the ecological and functional dynamics of gut environments. Honey bees are important pollinators of many crops for human consumption and produce valuable commodities such as honey and wax, making the gut microbiota of honey bees an interesting community to explore (Romero *et al*. 2019). Gut bacteria perform a wide variety of actions that have crucial implications for the host metabolism and overall health (Figure 1). Gut microbial populations are vital for many host organisms, from breaking down indigestible carbohydrates to immunomodulation (Hall *et al*. 2017).

Honey bees and their bumble bee relatives are globally important as they pollinate a vast majority of the world's crops and wild flowering plants. The recent decline in bee population has put a spotlight on potential agents that affect their health, including their microbiota (Cox-Foster *et al*. 2007; Furst *et al*. 2014; Crotti *et al*. 2013). Indeed, there is evidence that bacteria in the bee gut aid in defence against invading pathogens in ways that are still unclear (Koch and Schmid-Hempel 2011; Koch and Schmid-Hempel 2012).

Honey bees are fascinating and vivid creature with complex social system that has close interaction with their surrounding environment making them superorganisms. Fossil records indicate that among any other living species of *Apis*, *Apis florea* is the most primitive. This is substantiated by their colony size, open nest and behavioural pattern (Kaspi and Shafir 2013; Radloff *et al*. 2005). The evolutionary divergence of *Apis florea* from the common ancestor shows it to be much older than the remaining *Apis* clades (Biewer *et al*. 2016). These are prevalent and restricted to southern and south-eastern parts of Asia. It is commonly known as dwarf bee or little bee and endures temperature up to 50 °C (Biewer *et al*. 2016). These bees produce honey in smaller quantity and often consume it, and hence are not reared for commercial activity. Their contribution to pollination is agriculturally and ecologically significant rather than their role in producing honey. Depending upon forage availability, *A. florea* often migrates between plains and adjacent

low-lying slopes during different seasons. The species prefers warmer climate for their foraging activity (Balachandra *et al*. 1999) and functions as a significant pollinator.

The functions of the gut bacteria are delineated by studying the interactions among gut bacteria and the host. Gut bacterial diversity in honey bee belonging to *A. mellifera* (Western honey bee) is well demonstrated. Different social corbiculate bees possess highly characteristic gut communities. Invertebrate animal hosts provide models that are more tractable since their gut microbial populations are often simple. These microbial communities, however, are often variable in their composition and dominated by opportunistic environmental microorganisms. Rather than by specialised microbial symbionts that are specific to the host environment, like those found in mammalian guts (Kwong and Moran 2016; Engel and Moran 2013b; Chandler *et al*. 2011). These distinct gut communities inside the host are in large part independent of the geographical occurrence of the host (Kwong *et al*. 2017; Moran *et al*. 2012; Martinson *et al*. 2011). The composition of the microbiota in the adult gut changes little, despite seasonal variation and modification in nutrition, behaviour and gene expression that occur when workers transition from nursing bees to foragers (Martinson *et al*. 2012; Corby-Harris *et al*. 2014). However, high strain-level diversity is reported within the gut microbiota of *A. mellifera* and *A. cerana*, providing insights into the gut microbiota functioning and host-symbiont interactions (Ellegaard *et al*. 2020).

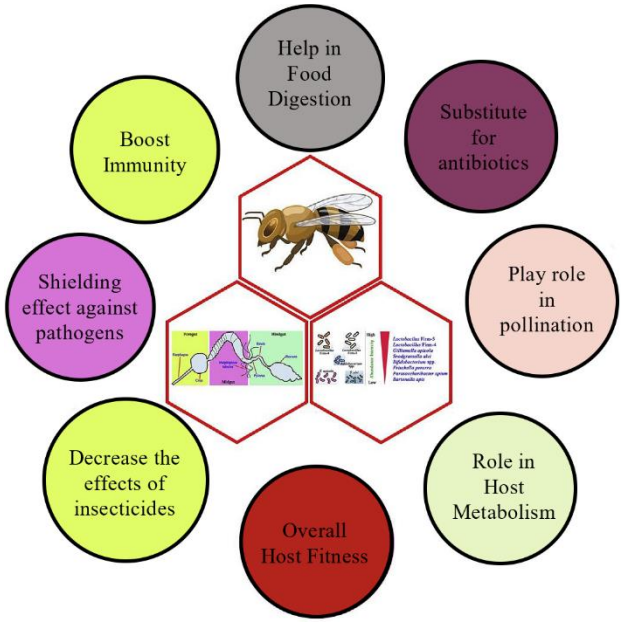

Figure 1: Honey bee gut associated microbiota and its functions

According to 16S rRNA gene analyses, eusocial corbiculate bees (honey bees, stingless bees and bumble bees) have a relatively simple, yet specialized, gut microbiota with a highly conserved taxonomic composition (Kwong *et al*. 2017; Kwong and Moran 2016). Some of these phylotypes are found in a wide range of social bees, indicating that they evolved along with eusociality in bees (Kwong *et al*. 2017). The diversity in the gut bacterial community among relative host species of honey bees represents major functional differences. The gut microbiota of social bees is an emerging model to study genomic diversity and evolution of host-associated bacterial communities because of these properties (Ellegaard and Engel 2019; Kwong *et al*. 2017; Kwong and Moran 2016; Ellegaard and Engel 2016; Ellegaard *et al*. 2020).

The microbiota is concentrated in the distal part of the gut, where it helps in the digestion and fermentation of plant cell wall components. Many bee gut bacteria are specific to the bee gut and can be transmitted directly between individuals through social interaction, similar to the human gut microbiota (Zheng *et al*. 2018). Gut bacteria are primarily transferred through social interactions in both bees and primates (Powell *et al*. 2016; Moeller *et al*. 2016; Tung *et al*. 2015). Many invertebrate gut communities, on the other hand, have unpredictable compositions dominated by bacteria from environmental sources (Wong *et al*. 2013; McFrederick *et al*. 2017).

The reared species of honey bee have occupied a dominant position in commercial pollination around the world, as these are highly social bees. Yet, on the other hand, wild bees are useful pollinators, too. Perhaps we have always under measured their contribution due to our limited insight into their behavioural mechanism for nesting. The other hypothesis may be that more efficient are the easily manageable honey bees that also provide by-products.

Variation in the composition of gut microbiota of worker bees is negligible despite shifts in host nutrition, behavioural pattern and gene expression (Martinson *et al*. 2012; Corby-Harris *et al*. 2014). The gut microbial communities of invertebrates are relatively simple and harbour dominant opportunistic environmental microorganisms rather than host-specific microbial symbionts (Kwong and Moran 2016; Engel and Moran 2013a; Chandler *et al*. 2011). The bacterial communities of wild flies are more diverse and differ from those of reared species (Chandler *et al*. 2011), including bee gut bacteria (Kwong and Moran 2016; Engel *et al*. 2013).

Insect diets are rich in plant polysaccharides, but generally lack the enzymes to metabolise these substrates. Rather, many rely on gut bacteria to break down polysaccharide bonds and release sugars or short-chain fatty acids that can be consumed by hosts. Dietary polysaccharides influence gut microbial ecology and, in turn, host physiology and health in humans. Honey bees have a similar bacterial community in the distal gut that can digest polysaccharides (HJ *et al*. 2008). Bees acquire carbohydrates and amino acids from nectar and pollen, which also includes polysaccharides like pectin, cellulose and hemicellulose. Through the microbial enzymatic activity, these potential energy sources could be metabolized, resulting in simple sugars available to hosts. However, the contribution of individual microbiota members to polysaccharide digestion has remained unclear (Zheng *et al*. 2019).

Considering the diet of bees that is rich in carbohydrates (consisting of nectar, honey and pollen), it is not surprising that gut microbiota comprises members that coevolved to thrive on this resource. All bee-associated bacteria can metabolise glucose and fructose, the most abundant sugars in the diet of honey bees. Furthermore, certain strains possess genes for the utilization of rarer sugars (pectin, cellulose, lignin, galactose and lactose), that are indigestible and potentially toxic to the bee (Kwong and Moran, 2016). The commercial value of various enzymes has increased over the last two decades, especially in conventional fields such as the food and detergent industries, but it is still improving, especially in areas like pollution control, medical applications, and biosensors. The global enzyme market is estimated to be worth around US$ 1.5 billion and growing at a rate of 5-10% per year (Munn, 2004). Only a few studies have been carried out in detail to elucidate the capacity of bee gut bacteria to produce extracellular enzymes (Fenice *et al*. 2007).

The discovery of novel genes and enzymes from different natural sources has recently become a subject of both basic and applied biotechnology research. Soil and other niches in the ecosystem were thought to be significant sources of novel biomolecules *(*Krishnan *et al*. 2014). The multienzymes activities of bacterial isolates from the bee gut could provide a potential source of enzymes for biotechnological applications (Zheng *et al*. 2019).

Unfortunately, the majority of microorganisms are unculturable, and hence cannot be analyzed using culture-based techniques. Culture-independent techniques have recently been successful in gaining access to previously unexplored genomic resources. However,

culture-based approaches cannot fully exploit such resources because the vast majority of microorganisms are unculturable in the laboratory (Zapka *et al*. 2017).

The microbiome of the majority of wild honey bee species have a complex composition and functions and must be elucidated (Engel *et al*. 2016). The demand for such a study emerges since the gut microbiome plays a critical role in the host health, and it shows the relationship between host health in both managed and wild pollinator bees (Engel *et al*. 2016). Using targeted amplicon sequencing, we attempted to profile the bacterial community composition of *A. cerana indica* and *A. florea* from the Indian subcontinent. We selected two distinct bee species from wild and reared habitats. Despite surging reports on gut microbiota, there is a lack of information on the gut microbiota of *A. florea*. Since *A. florea* is an essential contributor to crop pollination, the aim of this study was to explore and characterize the gut microbiota in the whole alimentary canal of *A. florea*. For a culture-based approach, isolates were subjected to MALDI-TOF-MS and 16S rRNA sequencing and to characterize the non-culturable organisms, a high-throughput sequencing technique was used (Figure 2). The study provides insights into this primitive clade of *Apis*. This investigation adds to the knowledge on bee microbiome to unravel the evolution and ecology of host–microbiome interactions (Engel *et al*. 2016).

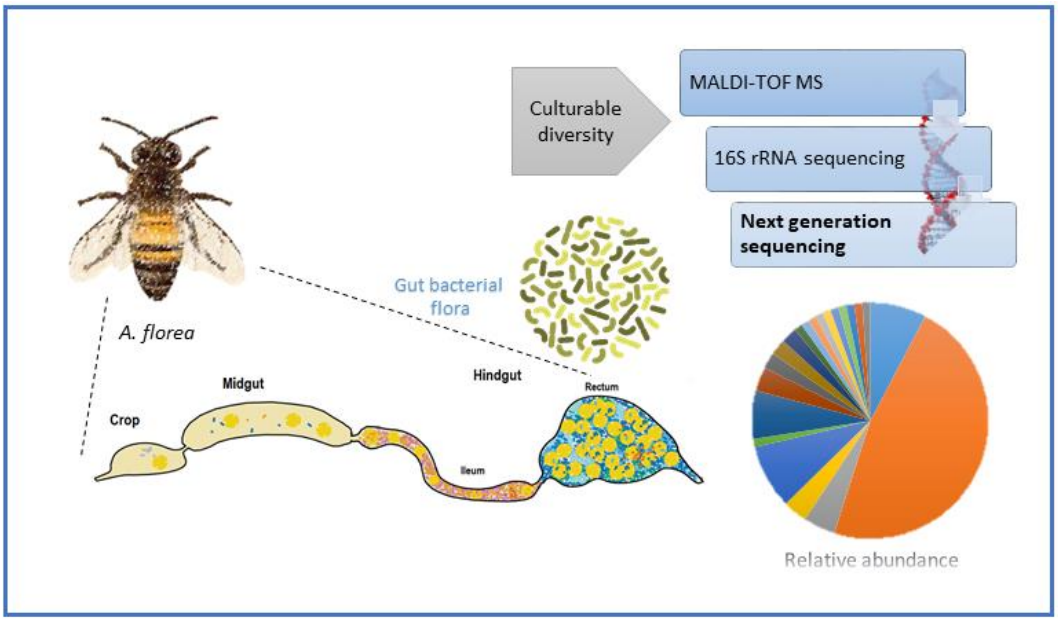

Figure 2: The composition of the gut bacterial flora in *A. florea* adult worker bees, based on cultivable and uncultivable community profile using MALDI TOF MS, 16S rRNA sequencing and Next generation sequencing techniques from complete alimentary canal.

The present study was primarily intended to find new strains for potential applications, but it may also partly contribute to the knowledge of the nutritional versatility of the isolates, and to some extent, their ecological importance. The gut-associated bacteria of *A. florea* produce polysaccharide degrading enzymes. The presence of carbohydrate degrading microorganisms, which contribute to host nutrition, is an important factor. In the present study, the potential bacterial strains were screened for the carbohydrate degrading enzymes from the honey bee gut microbiota. *Klebsiella oxytoca* af-G4, the dominant gut inhabitant of *A. florea* demonstrated the presence of significant pectinase and amylase activity. Production of pectinase and amylase using agro industrial wastes was carried out to find out the cost-economic sources. Here, we purified and characterized the enzyme pectinase by bacteria *K. oxytoca* af-G4.

The main objectives of the present investigation are:

1. **To study the gut microbial diversity of Asian honey bees**
2. **Identification of different carbohydrate degrading enzymes expressed by the honey bee gut microbiome**
3. **Characterization of carbohydrate degrading enzymes expressed by the honey bee gut microbiome**

The following studies were performed towards achieving these objectives:

(i) The identification and characterization of culturable and unculturable diversity of bee gut bacteria by employing MALDI-TOF-MS, 16S rRNA gene-sequencing and high-throughput sequencing approach (Next generation sequencing-NGS).

(ii) Functional analysis of bacterial communities to perform imputed metagenomics by using PICRUSt2 tool.

(iii) Screening of bacterial isolates to assess polysaccharide hydrolyzing enzymes.

(iv) Optimization of growth conditions using synthetic sources and agro-industrial wastes for the production of polysaccharide hydrolyzing enzymes by *Klebsiella oxytoca* af-G4 isolated from gut of honey bee, *Apis florea*.

(v) Purification and characterization of pectinase from *Klebsiella oxytoca* af-G4.

The thesis is organized into four chapters:

Chapter 1- Introduction

This chapter deals with the general introduction and scope of the present investigation. Literature review related to ecological and functional dynamics of gut environments and their role in honey bee health and nutrition. Significance of gut microbial population towards the host including breaking down indigestible carbohydrates to immunomodulation is described. The ability of bee gut bacteria to produce multiple polysaccharide degrading enzymes and their role in metabolism of honey bees, prominence of carbohydrases in various industries, production of carbohydrases using cost-effective sources such as agro industrial wastes and enzyme kinetics are detailed. This chapter ends with the aim and scope of investigation.

Chapter 2- Materials and Methods

Chapter 2 explain the experiments conducted to gather requisite data to fulfill the objectives of this study. Isolation, identification and characterization of culturable diversity of honey bee, *Apis florea* gut bacteria were carried out by employing MALDI-TOF-MS and 16S rRNA gene-sequencing analysis by using colony PCR. In the culture-independent study, a comprehensive gut microbial diversity analysis of *Apis cerana indica* and *A. florea* was carried out using high- throughput sequencing (NGS) approach and alpha diversity indices were assessed by richness like Chao1 and ACE, and diversity (Shannon) was calculated via QIIME. The sequences obtained from both culture-dependent and culture-independent approaches were submitted to NCBI database. Functional analysis of bacterial communities was performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) tool. Screening of bacterial isolates to assess their polysaccharide hydrolyzing enzyme activity was performed. Production and optimization of growth parameters for amylase and pectinase by *Klebsiella oxytoca* af-G4 isolated from honey bee, *Apis florea* gut was performed. Effect of different agro industrial wastes and optimization of growth conditions in pectinase and amylase production were carried out. Purification and characterization of pectinase from *Klebsiella oxytoca* af-G4 were performed.

Chapter 3- Results and Discussion

This chapter deals with the results and discussion of the investigation under six sections (1 – 6).

Section 1. Characterization of gut bacterial culturable diversity of *A. florea* by employing MALDI-TOF-MS and 16S rRNA gene-sequencing

In the culture-dependent approach, the identification and characterization of culturable diversity of bee gut bacteria was done by employing MALDI-TOF-MS and 16S rRNA gene-sequencing analysis by colony PCR. A total of 146 aerobic and facultative anaerobic bacteria were isolated from the guts of worker *A. florea* bees. Based on colony morphology and other bacteriological characteristics, 106 bacterial isolates were initially subjected to MALDI-TOF-MS. Seventy-one isolates were identified up to species level with biotyper score value ≥2.0. However, isolates with biotyper score value <2.0 could not be identified up to species level. The remaining thirty-five isolates were subjected to 16S rRNA sequencing using colony PCR. MALDI-TOF-MS and 16S rRNA sequencing of the culture-dependent analysis identified three bacterial phyla/classes: alpha-Proteobacteria (1%), Firmicutes (16%) and gamma-Proteobacteria (83%). The percentage of isolates belonging to genera *Klebsiella, Enterobacter, Bacillus, Citrobacter, Staphylococcus* and *Lactobacillus* are represented.

The sequences obtained from Culture-dependent approach was submitted to NCBI, which are available under Bio Project SUB6349615, MN512276:MN512310 [accn].

Section 2. Culture-independent gut microbial diversity analysis of *Apis cerana indica* and *A. florea* using high-throughput sequencing (NGS)

Characterization of microbial diversity

In both *A. florea* and *A. cerana indica*, high-throughput sequencing and quality trimming of the 16S rRNA gene yielded ~0.29 million quality reads, which were used for subsequent data analysis. Sequences were taxonomically assigned using the reference database, yielding 867 operational taxonomic units (OTUs). When computed for bacterial diversity, alpha diversity estimation of the guts of *A. florea* and *A. cerana indica* using species richness and non-parametric shannon index revealed substantial differences between them. The bacterial species richness of *A. florea* (850.84) worker bees was found to be higher than that of *A. cerana indica* (498.60). The non-parametric shannon index for bacterial

communities also differed between *A. florea* gut (3.12) and *A. cerana indica* gut (3.29). This finding suggests that the gut of *A. cerana indica* has more bacterial diversity than *A. florea*. This observation showed that both the species shared 36.3% OTUs at genus level.

Bacterial community profiles in the guts of *A. florea* and *A. cerana indica*

16S rRNA gene amplicon sequencing yielded 16 and 12 bacterial phyla in the gut of *A. cerana indica* and *A. florea*, respectively. In *A. cerana indica* gut bacterial phyla distributions were Proteobacteria (50.09%), Firmicutes (26.29%), Bacteroidetes (23.19%), Actinobacteria (0.4%), and the rest (0.03%) constituted the minor phyla Acidobacteria, Euryarchaeota, Cyanobacteria, Chloroflexi, Saccharibacteria, Verrucomicrobia, Planctomycetes, Gemmatimonadetes, Thaumarchaeota, Armatimonadetes, FBP and Tenericutes. The distributions of bacterial phyla in the gut of *A. florea*, were as follows: Proteobacteria (86.86%), Firmicutes (12.81%), Actinobacteria (0.27%), Bacteroidetes (0.04%) and the rest (0.02%) constituted the minor phyla Acidobacteria, Cyanobacteria, Chloroflexi, Verrucomicrobia, Planctomycetes, Nitrospirae, Lentisphaerae and Tectomicrobia. Bacterial phylum Proteobacteria outnumbered other bacterial phyla in the gut of *A. florea*, whereas *A. cerana indica* gut was found to constitute Proteobacteria, Firmicutes and Bacteroidetes as major phyla.

At family level, OTUs with ≥0.1% abundance were filtered which differed quantitatively between *A. cerana indica* and *A. florea*. The family-level distributions of bacteria in the gut of *A. cerana indica* were Lactobacillaceae (26.39%), Flavobacteriaceae (23.21%), Orbaceae (19.12%), Neisseriaceae (16.3%), Oxalobacteraceae (13.91%), Enterobacteriaceae (0.53%), Bifidobacteriaceae (0.27%), Moraxellaceae (0.15%) and Nocardiaceae (0.12%). Whereas the family-level distributions of bacteria in the gut of *A. florea* were Enterobacteriaceae (79.47%), Lactobacillaceae (12.75%), Oxalobacteraceae (7.45%), Nocardiaceae (0.13%), Micrococcaceae (0.07%), Bifidobacteriaceae (0.05%), Staphylococcaceae (0.04%) and Prevotellaceae (0.04%).

At the genus level, OTUs with ≥0.1% abundance were filtered and distributions of bacterial genera in the gut of *A. cerana indica* were *Lactobacillus* (26.41%), *Apibacter* (23.22%), unidentified bacteria (20.58%), *Massilia* (13.92%), *Orbus* (11.86%), *Gilliamella* (3.08%), *Bifidobacterium* (0.26%), *Escherichia-Shigella* (0.24%), *Rhodococcus* (0.12%) and *Alkanindiges* (0.11%). Genera in *A. florea* gut were *Enterobacter* (58.49%), *Lactobacillus* (14.47%), *Escherichia-Shigella* (13.29%), *Massilia* (8.45%), *Klebsiella* (1.71%),

Citrobacter (1.35%), *Pantoea* (1.32%), *Serratia* (0.63%), *Rhodococcus* (0.15%) and *Morganella* (0.13%).

The sequencing reads generated during high throughput sequencing effort, are available under NCBI Bio Project ID: PRJNA777397.

Section 3. Functional imputations of bacterial communities was performed using PICRUSt2 tool.

Functional prediction of the gut bacterial communities of *A. florea* and *A. cerana indica* using PICRUSt2 revealed higher metabolism-related genes, inferring that these bacterial populations play a significant role in macromolecule digestion. Furthermore, genes involved in environmental information processing, genetic information processing, and cellular activities were found.

Expansion of each functional modules such as metabolism is correspondingly prevalent in carbohydrate metabolism (32.4%), followed by amino acid metabolism (16.43%), cofactor and vitamin metabolism (12.77%), nucleotide metabolism (11.13%), energy metabolism (9.1%), and lipid metabolism (9.1%). (5.6 %). Similarly, membrane transport (64.65%), translation (48.53%), and cellular community – Prokaryotes (43.92%) were identified as significant contributors to environmental information processing, genetic information processing, and cellular processes, respectively.

Section 4. Screening of bacterial isolates to assess their polysaccharide hydrolyzing enzyme activity

All the selected isolates exhibited amylase and pectinase activities. However, only two isolates exhibited ligninolytic and cellulolytic activity. None of the isolates showed tannin degradation. *Bacillus halotolerans* af-M9 (4.95 U/mg) and *Klebsiella oxytoca* af-G4 (4.62 U/mg) showed the maximum amylase activity amongst others. With respect to pectinase activity *Klebsiella oxytoca* af-G4 (0.20 U/mg) and *Klebsiella oxytoca* af-M1 (0.15 U/mg) displayed higher activity.

Section 5. Production and optimization of growth parameters for amylase and pectinase by *Klebsiella oxytoca* af-G4 isolated from honey bee, *Apis florea* gut

The optimization of growth parameters for the pectinase production in both synthetic source and agro industrial waste were studied and the results are as follows,

Synthetic source: Nutrient broth (pH 5) supplemented with filter sterilized lactose (1%) and ammonium sulphate (1%) was inoculated with *Klebsiella oxytoca* af-G4 culture (1%) and incubated at 30 °C showed maximum pectinase activity. At regular interval of time (24, 48 and 72 h), an aliquot was drawn and checked for bacterial growth and pectinase activity. Maximum pectinase activity (3.23 U/mg) was observed at 72 h incubation time provided with above mentioned optimized conditions.

Agro industrial waste: Nutrient broth adjusted to pH 6 was supplemented with 1% pomegranate peel and inoculated with *Klebsiella oxytoca* af-G4 culture (1%). The conical flask was incubated at 40 °C. At regular interval of time (24, 48, 72, 96, 120 h), an aliquot was drawn and checked for bacterial growth and pectinase activity. Maximum pectinase activity (3.5 U/mg) was observed at 96 h incubation time provided with above mentioned optimized conditions.

Section 6. Purification and characterization of pectinase from *Klebsiella oxytoca* af-G4

The *Klebsiella oxytoca* af-G4 culture was grown in 1L of optimized synthetic media under specified conditions. Ammonium sulpahte precipitation was carried out at 75% saturation and dialyzed. The dialyzed sample (50 mg protein) was loaded onto a DEAE cellulose column (18X3 cm) pre-equilibrated with 0.2 M sodium phosphate buffer (pH 7). Maximum protein content and pectinase activity was observed in fraction 2 eluted at linear gradient of 0.2 M NaCl in 0.2 M sodium phosphate buffer. The crude, ammonium sulphate precipiate and column fraction (showing highest activity) was analyzed on SDS-PAGE. Column fraction was also checked for activity by zymogram. The purity of pectinase was confirmed by single band in SDS-PAGE. The purified enzyme is showing Mw. ~60 kDa, activity 23.26 U/ml and specific activity of 14.36 U/mg. The purified enzyme exhibited optimum activity at a temperature of 60 °C and pH 6. The maximum activity of pectinase from *Klebsiella oxytoca* af-G4 was obtained using 200 µl of enzyme. The enzyme was stable within pH range of 5 to 8 with optimum pH of 6. A further decrease in pH led to a marked decrease in stability of the enzyme. The enzyme was found stable within temperature range of 30 °C to 80 °C and above this range >80 °C indicating the instability of the enzyme. The K_m and V_{max} values of pectinase were found to be 6.04 mg/ml and 4.92 U/ml, respectively, at different concentrations of the substrate, that is, 0.1% to 0.5% (w/v) by plotting the Lineweaver-Burke plot. The enzyme showed 15-20% decrease in activity

in presence of Ca^{2+}, $[NH_4]^+$, K^+ and Na^+ whereas Mn^{2+} caused 60% increase in the enzyme activity. Thus, pectinase from *Klebsiella oxytoca* af-G4 can be exposed for its potential application such as juice clarification, textile, plant fiber processing, tea, coffee, oil extraction, and treatment of industrial waste water containing pectinaceous material.

Chapter 4- Summary and Conclusions

The gut communities of *A. florea* and *A. cerana indica* are more diverse in composition compared to other *Apis* clades. Most of the isolates observed are opportunistic and beneficial gut inhabitants, which is distinct in abundance in opposition to other species of *Apis*. Functional analysis revealed the highest abundance of metabolism-related genes in bacterial communities of both the bee species. This work provides additional information and data pertaining to the gut microbiome of honey bees. The cultured bacterial isolates from *A. florea* gut showed multiple polysaccharide degrading abilities. The enzyme pectinase from *K. oxytoca* af-G4 possess superior properties including high temperature tolerance and being active in acidic/alkaline conditions. The enzyme plays a potential role in juice extraction and clarification and this enhances its possible application in various industries and could be used as a substitute for the commercial pectinase production. However, further genomic investigations are required to understand the significance of diverse gut microbial populations of managed and wild honey bee species, enzymes derived from gut microbes and their potential in various industries.

Bibliography

All the references followed by publications from the present investigation will be listed at the end of four chapters.

References

Balachandra C, Subash Chandran MD, Ramachandra TV. Honeybee Diversity, Role in Pollination and Beekeeping Scenario in South Indian Western Ghats, 1999.

Biewer M, Lechner S, Hasselmann M. Similar but not the same: Insights into the evolutionary history of paralogous sex-determining genes of the dwarf honey bee *Apis florea*. Heredity (Edinb) 2016; 116: 12–22. https://doi.org/10.1038/hdy.2015.60.

Chandler JA, Lang JM, Bhatnagar S, Eisen JA et al. Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet 2011; 7: e1002272.

Corby-Harris V et al. Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov. Appl. Environ. Microbiol 2014; 80, 7460–7472.

Cox-Foster DL et al. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 2007; 318, 283–287.

Crotti E et al. Microbial symbionts of honeybees: a promising tool to improve honeybee health. N. Biotechnol 2013; 30, 716–722.

Ellegaard KM, Engel P. Beyond 16S rRNA community profiling: intra-species diversity in the gut microbiota. Front. Microbiol 2016; 7, 1475.

Ellegaard KM, Engel, P. Genomic diversity landscape of the honey bee gut microbiota. Nat. Commun 2019; 10, 446.

Ellegaard KM, Suenami S, Miyazaki R, Engel P. Vast Differences in Strain-Level Diversity in the Gut Microbiota of Two Closely Related Honey Bee Species. Curr Biol 2020: 1–12. https://doi.org/10.1016/j.cub.2020.04.070.

Engel P, Kwong WK, McFrederick Q, Anderson KE et al. The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions. mBio 2016;7(2):e02164-15, doi:10.1128/mBio.02164-15.

Engel P, Kwong WK, Moran NA. *Frischella perrara* gen. nov., sp. nov., a gammaproteobacterium isolated from the gut of the honey bee, *Apis mellifera*. Int J Syst Evol Microbiol 2013, doi: 10.1099/ijs.0.049569-0.

Engel P, Moran NA. Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. Gut Microbes 2013a; 4: 60–65. https://doi.org/10.4161/gmic.22517.

Engel P, Moran NA. The gut microbiota of insects - diversity in structure and function. FEMS Microbiol Rev 2013b; 37: 699–735. https://doi.org/10.1111/1574-6976.12025.

Fenice M, Gallo AM, Juarez-Jimenez B, Gonzalez-Lopez J. Screening for extracellular enzyme activities by bacteria isolated from samples collected in the Tyrrhenian Sea. Ann. Microbiol. 2007 571 57, 93–99. https://doi.org/10.1007/BF03175056

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol. 2008 Feb;6(2):121-31. doi: 10.1038/nrmicro1817. PMID: 18180751.

Furst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJ. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 2014; 506, 364–366.

Hall AB, Tolonen AC, Xavier RJ. Human genetic variation and the gut microbiome in disease. Nat Rev Genet. 2017 Nov; 18(11): 690-699. doi: 10.1038/nrg.2017.63. Epub 2017 Aug 21. PMID: 28824167.

Kaspi R, Shafir S. Associative olfactory learning of the red dwarf honey bee *Apis florea*. Apidologie 2013; 44: 100-109.

Krishnan M, Bharathiraja C, Pandiarajan J, Prasanna VA, Rajendhran J, Gunasekaran P. Insect gut microbiome - An unexploited reserve for biotechnological application. Asian Pac. J. Trop. Biomed 2014. 4, S16–S21. https://doi.org/10.12980/APJTB.4.2014C95

Koch H, Schmid-Hempel P. Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host–parasite system. Ecol. Lett. 2012; 15, 1095–1103.

Koch H, Schmid-Hempel P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc. Natl Acad. Sci. USA 2011; 108, 19288–19292.

Kwong WK, Medina LA, Koch H, Sing KW et al. Dynamic microbiome evolution in social bees. Sci Adv 2017; 3: 1–17. https://doi.org/10.1126/sciadv.1600513.

Kwong WK, Moran NA. Gut Microbial Communities of Social Bees. Nat. Rev. Microbiol. 2016; 14, 374. https://doi.org/10.1038/NRMICRO.2016.43

Martinson VG, Danforth BN, Minckley RL, Rueppell O et al. A simple and distinctive microbiota associated with honey bees and bumble bees. Mol Ecol 2011; 20: 619–628.

Martinson VG, Moy J, Moran NA. Establishment of characteristic gut bacteria during development of the honeybee worker. Appl. Environ. Microbiol 2012; 78: 2830–2840. https://doi.org/10.1128/AEM.07810-11.

McFrederick QS et al. Flowers and wild megachilid bees share microbes. Microb. Ecol. 2017; 73, 188–200.

Moeller AH et al. Cospeciation of gut microbiota with hominids. Science 2016; 353, 380–382.

Moran NA, Hansen AK, Powell JE, Sabree ZL. Distinctive Gut Microbiota of Honey Bees Assessed Using Deep Sampling from Individual Worker Bees. PLoS One 2012;7: e36393.

Munn C. 2004. Microbes in the Marine Environment, Marine Microbiology Ecology and Applications. Garland Science/BIOS Scientific Publishers ; Distributed in the USA by Fulfilment Center, Taylor & Francis, London ; New York, N.Y. Independence, KY.

Powell JE, Martinson VG, Urban-Mead K, Moran NA. Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. Appl. Environ. Microbiol. 2014; 80, 7378–7387.

Radloff S, Hepburn H, Hepburn C et al. Multivariate morphometric analysis of *Apis cerana* of

southern mainland Asia. Apidologie 2005; 36(1): 127-139. doi: 10.1051/apido:2004077.

Romero S, Nastasa A, Chapman A, Kwong W et al. The honey bee gut microbiota: strategies for study and characterization. Insect Molecular Biology 2019; 28(4): pp.455-472.

Tung J et al. Social networks predict gut microbiome composition in wild baboons. eLife 2015; 4, 305224.

Wong AC, Chaston JM, Douglas AE. The inconstant gut microbiota of Drosophila species revealed by 16S rRNA gene analysis. ISME J. 2013; 7, 1922–1932.

Zapka C, Leff J, Henley J, Tittl J, De Nardo E, Butler M, Griggs R, Fierer N, Edmonds-Wilson S. Comparison of standard culture-based method to culture-independent method for evaluation of hygiene effects on the hand microbiome. MBio 2017; 8. https://doi.org/10.1128/MBIO.00093-17

Zheng H, Perreau J, Elijah Powell J, Han B, Zhang Z, Kwong WK, Tringe SG, Moran NA. Division of labor in honey bee gut microbiota for plant polysaccharide digestion. Proc. Natl. Acad. Sci. U. S. A 2019; 116, 25909–25916. https://doi.org/10.1073/pnas.1916224116

Zheng H, Steele M, Leonard S, Motta E, Moran N. Honey bees as models for gut microbiota research. Lab Animal 2018; 47(11), pp.317-325.

Original research articles of this work published in scientific journals

1. **Ganeshprasad DN**, Karthik Yalpi, Sachin HR, Sneharani AH. Polysaccharide hydrolyzing enzyme activity of bacteria, native to *Apis florea* gut. *Biomedicine*. 2021; 41(4):768-775.
2. **Ganeshprasad DN**, Lone JK, Jani K, Shouche YS, Khan KA, Sayed S, Shukry M, Dar SA, Mushtaq M and Sneharani AH (2022) Gut Bacterial Flora of Open Nested Honeybee, *Apis florea*. *Front. Ecol. Evol.* 10:837381. doi: 10.3389/fevo.2022.837381.
3. **D.N. Ganeshprasad**, Kunal Jani, Yogesh S. Shouche, A.H. Sneharani. Variation in gut bacterial diversity of two Asian honey bees, *Apis cerana indica* and *Apis florea* (Communicated).
4. **D.N. Ganeshprasad** and A.H. Sneharani. Pectinase production, purification and characterization from *Klebsiella oxytoca* af-G4 isolated from honey bee, *Apis florea* gut. *Journal of King Saud University* (Under Review).

Conference papers

1. **Ganeshprasad D.N.,** Yogesh S. Shouche and A.H. Sneha Rani., Characterization of gut bacterial flora of *Apis florea* from south Indian sub-continent. International conference on "Impact of Innovations in science and technology for societal development (IISTSD-2019).
2. **Ganeshprasad D.N.**, Kunal Jani, R. N. Kencharaddi, Yogesh S. Shouche and A.H. Sneha Rani., Variation in gut bacterial diversity of two Asian honey bees, *Apis cerana indica* and *Apis florea*. "Advances in Biotechnology & Bioinformatics, Research Scholars Meet (Online) – 2021" (RSM-2021).
3. **Ganeshprasad D.N.** and A.H. Sneha Rani., Purification and Characterization of Pectinase from Gut-Associated *Klebsiella oxytoca* af-G4 of honey bee, *Apis florea*. National conference on "Impact of Research Development in Life Sciences" IRDLS-2022.
4. **Ganeshprasad D.N.**, Kunal Jani, Yogesh S. Shouche and A.H. Sneha Rani. Analysis of Gut Microbial Diversity of two closely related Honey Bee Species" "National conference on Integrated Microbial Technology for Sustainable future" IMTSF-2022.

Publications Co-authored

1. Sachin, H.R, S.H. Prabhudev, **D.N. Ganeshprasad**, and A.H. Sneharani. "Immobilization of Trypsin Enzyme on Silver Nanoparticles". Biomedicine, vol. 40, no. 2, Nov. 2020, pp. 188-91, doi:10.51248/.v40i2.66.

Mr. Ganeshprasad D. N.
(Research Scholar)

Dr. Sneha Rani A. H.
(Research Supervisor)

Date: -08-2022
Place: Chikka Aluvara